

AD _____

Award Number: DAMD17-03-1-0067

TITLE: Microlocalization and Quantitation of Risk Associated
Elements in Gleason Graded Prostate Tissue

PRINCIPAL INVESTIGATOR: Curtis Eckhert, Ph.D.

CONTRACTING ORGANIZATION: University of California
Los Angeles, CA 90024

REPORT DATE: March 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20040903 041

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE March 2004	3. REPORT TYPE AND DATES COVERED Annual (1 Mar 2003 - 28 Feb 2004)	
4. TITLE AND SUBTITLE Microlocalization and Quantitation of Risk Associated Elements in Gleason Graded Prostate Tissue			5. FUNDING NUMBERS DAMD17-03-1-0067	
6. AUTHOR(S) Curtis Eckhert, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California Los Angeles, CA 90024 E-Mail: ceckhert@ucla.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) Epidemiological and laboratory studies show that boron, selenium and zinc reduce prostate cancer risk whereas calcium and cadmium increase risk. The objective of this proposal is to determine the concentration and location of these elements in normal and tumor tissue. Specific aims include: (1) preparation of four grades of prostate tissue, (2) determination of tissue concentrations of: B, Ca, Cd, Se, and Zn; and (3) determination of tissue and cellular distribution of these elements using NanoSIMS ion microscope at Lawrence Livermore National Laboratory (LLNL). Progress toward specific aim 1 includes preparing matched samples of normal tissue with graded tumor tissue from pathological tissue at prostatectomy. Progress toward specific aim 2 includes method development and analysis of 23 paired samples of normal and Gleason graded tumor tissue. Results were submitted in October for presentation at the April 2004 Experimental Biology Meetings in Washington, D.C. The data do not support an association between Gleason scores and the elements concentration in prostate tissue, but show considerable variability in the population. Work toward specific aim 3 has concentrated on methods development for NanoSIMS ion microscopy. Two students will undergo training at LLNL to become expert in the use of the NanoSIMS ion microscopy.				
14. SUBJECT TERMS Prostate cancer, Gleason grade, risk factors, nutrition, nutritional science in cancer prevention, elements, quantitation, localization, biomarkers			15. NUMBER OF PAGES 12	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusions.....	8
References.....	8
Appendices.....	9

INTRODUCTION

There is growing evidence that the elements boron, selenium and zinc reduce PCa risk whereas calcium and cadmium increase risk (1-14). The objective of this proposal is to determine if these elements differ in concentration and location between normal and tumor tissue. The major goals of the first year of the project were to obtain pathologically graded human prostate; to determine the concentrations of boron, calcium, cadmium, selenium and zinc in these tissues; and to evaluate statistically the relationship between Gleason scores and elemental composition.

BODY

Task 1. To identify and maintain a series of progressively dedifferentiated samples (months 1-30).

a. Obtain specimens from radical prostatectomy from the UCLA Human Tissue Research Center.

Accomplishments: Unmatched and matched sets of normal and tumor prostate tissue have been collected from the UCLA Human Tissue Research Center. Unmatched samples were used to establish the methods for elemental analysis.

b. Develop four (donor/tissue) classification categories that reflect increased risk of prostate cancer (normal man/normal tissue; cancer patient/very mild dysplasia (Gleason's grade total 2-4), cancer patient/moderately differentiated adenocarcinoma (Gleason's grade total = 5-7) and poorly differentiated adenocarcinoma (Gleason's grade total = 8-10).

Accomplishments: We continue to obtain tissues in all categories for elemental analysis. We used normal tissue from men with cancer because samples of normal tissue from normal men are nearly none existent. The vast majority of tissue samples available have Gleason scores in the range from 6 to 7.

Task 2. To utilize state of the art inductive coupled plasma mass spectrometry to determine the concentration of the B, Ca, Cd, Se and Zn in whole tissue samples (Months 1-12).

a. Analyze the four classification categories of biopsy tissue by inductively coupled plasma mass spectrometry (ICPMS) to obtain whole tissue concentrations of: B, Ca, Cd, Se and Zn.

Accomplishments: We analyzed matched pairs of normal and tumor tissue to determine the concentrations of B, Ca, Cd, Se and Zn in 23 different male donors. The mean, median, range and coefficient of variation of the concentrations are given in Table 1.

Table 1. Elemental Concentrations of Normal and Tumor Prostate Tissue in 23 Men						
Normal		Boron	Calcium	Cadmium	Selenium	Zinc
		ng/g	μg/g	ng/g	ng/g	μg/g
	Mean	291	313	71	471	86
	Median	90	300	58	480	73
	Range	28 - 3530	82 - 830	8 - 220	200 - 730	9 - 190
	Std Error	159	36	10	24	11
	CV	256%	56%	66%	25%	63%
Tumor						
	Mean	355	263	83	502	88
	Median	100	270	61	520	84
	Range	23 - 2360	80 - 430	20 - 250	15 - 820	110- 180
	Std Error	129	18	12	34	9
	CV	170%	33%	70%	33%	50%

The concentrations in both normal and tumor tissue ranked as follows: Ca > Zn > Se > B > Cd. A statistical comparison of elemental concentrations in normal and tumor tissue did not reveal significant differences (Table 2). The coefficient of variation of the elements varied greatly between elements (Table 1). The magnitude of variation followed the same rank in normal and tumor tissue: B > Cd > Zn > Ca > Se. The high variation in boron concentrations was not expected. Boron is not known to activate or covalently bind to proteins. It's variability and 10 fold range in concentration suggests that prostate is able to accumulate boron. Examination of tissue using the NanoSIMS ion probe will determine where this occurs for boron and the other elements.

Table 2. Statistical Evaluation of Elemental Concentrations in Matched Normal and Tumor Tissue	
Element	Statistical Comparison between Normal and Tumor Tissue
Boron ¹	p = 0.31
Calcium ¹	p = 0.46
Cadmium ¹	p = 0.58
Selenium ¹	p = 0.21
Zinc ²	p < 0.09

- b. Determine the strength of the relationship between whole tissue elemental concentrations and pathological tissue classification will be determined by statistical analysis.

Accomplishments: Table 3 shows there was no relationship between Gleason score and the concentration of any element.

Table 3. Statistical Evaluation of the Relationship between Gleason Scores and Elemental Concentrations in Tumor Tissue	
Element	Correlation Coefficient of Gleason Score versus Element Concentration
Boron ¹	R = 0.10
Calcium ¹	R = 0.13
Cadmium ¹	R = 0.08
Selenium ¹	R = 0.30
Zinc ²	R = 0.21

Each of these elements has been associated with prostate cancer risk. The positive and negative associations with risk did not show up as concentration differences at the gross tissue level. The planned NanoSIMS measurements of the distribution and localization of the elements will provide a better measure of the role these elements might play in the etiology of prostate cancer.

Task 3. To determine the microlocation and microconcentrations of B, Ca, Cd, Se and Zn in graded series of samples (Months 6-36).

- a. Ion and photographic imaging of the four pathological categories of biopsy tissues will be accomplished using the ion microscope (NanoSIMS 50) located in the Analytical and Nuclear Chemistry Division of the Lawrence Livermore National Laboratory.
- b. Ion and photographic images will be analyzed using image analysis software to locate cellular sites of elemental accumulations in the four pathological categories.
- c. The relative concentrations of the elements at concentrated sites will be obtained in relationship to K and Na from the ion and photographic images using image analysis software.
- d. The spatial distribution and concentrations of elements will be used to determine if statistically meaningful correlations exist between the concentration of elements at specific cellular targets and the pathological classification of cancer.

e. Graphic representations will be used to show the relationship between concentrations in whole tissue as well as microcellular deposits of the elements, and the differentiation state of the tissue.

Accomplishments: Progress on Task 3 focused on the development of methods to prepare tissue for ion microscopy. We purchased a Leica EM MM80 slam freezer to ultra-rapidly freeze prostate tissue and a Thermo Neslab CC-100 cold probe to regulate the dehydration phase. Briefly, the Leica slam freezer was prechilled by filling with liquid nitrogen. The tissue was slam frozen and transferred to a Dewar containing acetone chilled to -83°C . The temperature of the acetone bath was controlled using the Thermo Neslab CC-100 probe and refrigeration unit. Samples were slowly brought to room temperature over a period of 72 hours. The dehydrated tissue was fixed in osmium and embedded in Spurr Low Viscosity Resin. This procedure has been successful using cultured prostate cells as the preparations look good when evaluated by scanning electron microscopy. We are currently tackling the more difficult task of slam freezing prostate tissues. The technician who embeds samples for the NanoSIMS at Lawrence Livermore is providing assistance. The NanoSIMS ion microscope was just installed at Lawrence Livermore in the Fall and two students will attend an eight week training session this summer to become expert in the use the instrument in cancer biology.

KEY RESEARCH ACCOMPLISHMENTS

- Successful procurement of Gleason graded tissue classified as normal, gleason grades 3, 5, 6, 7, 8.
- Analysis of matched sets of normal and tumor tissue obtained from 23 different men for the elements, boron, calcium, cadmium, selenium and zinc.
- Statistical analysis shows there is no difference between normal and tumor concentrations of these elements.
- Observed a 10 fold range of boron concentration and variation in prostate tissue. This suggests that the element is accumulated in prostate.

REPORTABLE OUTCOME

Work supported solely by this grant resulted in one abstract to the Experimental Biology Meetings (April 2004) and two other abstracts from students involved in working out methods for preparing tissue for NanoSIMS microscopy.

This project is providing the opportunity for two students to enter the Seaborg Training program at the Lawrence Livermore National Laboratory. The students will learn the theory and use of a range of sophisticated equipment in the Chemical and Physical Division and train to become experts in the use the NanoSIMS ion microscope.

CONCLUSIONS

The project is on schedule. During the first year work under task 1 was afforded the opportunity to work out the ICPMS procedures and progress to task 2. Work under Task 2 resulted in the preparation and elemental analysis of 23 matched pairs of normal/tumor prostate tissue. Work toward Task 3 was started and two students were accepted for training at the Lawrence Livermore National Laboratory in the use of the NanoSIMS ion microscope. Training will take place this summer as well as the first analyzes of prostate cancer tissue by ion microscopy. It is recommended that the project take advantage of the sophisticated equipment at both UCLA and LLNL to determine the molecular association of the elements under study. We recommend an elemental analysis of the proteomes as a way to determine if specific metalloproteins differ between normal and tumor tissue.

The following list of people received pay from the research effort.

Joey Miller (Student)
Wade Barranco (Student)
Curtis Eckhert (PI)

REFERENCES

1. Chan, JM, Stampfer MJ, Ma J, Gann, PH, Gaziano JM, Giovannucci EL. 2001. Dairy products, calcium, and prostate cancer risk in the Physicians' Health Study. *Am. J. Clin Nut.* 74:549-554.
2. Clark LC, Combs F, Turnbull BW, Slate EH, Chalker DK, et al. 1996. Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group. *JAMA* 276:1957-63.
3. Coffey DS. New insights and methodologies are needed to solve the many epidemiologic enigmas of prostate cancer. 2001. *Epidemiologic Reviews* 23, 1)
4. Feustel A, Wennrich R, Steiniger D, Klauss P. 1982. Zinc and cadmium concentrations in prostatic carcinoma of different histological grading in comparison to normal prostate tissue and adenofibromyomatosis (BPH) *Uro Res* 10:301-303.
5. Feustel A, Wennrich R. 1984 Zinc and cadmium in cell fractions of prostatic cancer tissues of different histological grading in comparison to BPH and normal prostate. *Urol Res* 12:147-50.
6. Gallardo-Williams MT, Maronpot RR, King PE, Moser GJ, Goldsworthy, TL, Chapin RE. Effects of boron supplementation on the morphology, PSA levels, and proliferative activity of LNCaP tumors in nude mice. 2002. *Proc Amer Assoc Cancer Res* 43:A388.
7. Greenwald P. 2001. Clinical trials of breast and prostate cancer prevention. *J. Nutr.* 131:176S-178S.
8. Hayes RB. 2001. Gene-Environmental interrelations in prostate cancer. *Epidemiologic Rev.* 23: 163-172.

9. Kantoff PW, Febbo PG, Giovannucci E, Krithivas K, Dahl DM, et al. 1997. Cancer Epidemiol. Biomarkers Prev. 6, 189-92.
10. Lindegaard PM, Hansen SO, Christensen JEJ, Andersen BB, Andersen O. 1989. The distribution of cadmium with the human prostate. Bio. Trace Ele Res 25:97-104.
11. Oldererid NB, Thomassen Y, Purvis K. 1998. Selenium in human male reproductive organs. Human Repro. 13:2172-76.
12. Tvedt KE, Kopstad G, Haugen OA Halgunset J. 1987. Subcellular concentrations of calcium, zinc, and magnesium in benign nodular hyperplasia of the human prostate: X-ray microanalysis of freeze-dried cryosections. Cancer Res 47:323-328.
13. Zhang Z-F, Winton MI, Rainey C Eckhert CD. 2001. Boron is associated with decreased risk of human prostate cancer. FASEB J 15:A834.4.
14. Cui Y, Winton M.I., Zhang, Z.F, Rainey C., Marshall J., deKernion J. B., Eckhert, C.D. Dietary Boron Intake and Reduced Risk of Prostate Cancer. *Oncology Reports* 11:887-892, 2004.

APPENDIX I

Eckhert CD. Concentration and Variation of Boron, Selenium and Elements Associated with Cancer Risk in Non-tumor Human Prostate Tissue. FASEB, 2004 (Abstract Experimental Biology Meeting April, 2004).

Barranco WT, Eckhert CD. Boric Acid Acts as a cADPR/RYR Antagonist During Inhibition of Human Prostate Cancer Cell Proliferation. FASEB, 2004 (Abstract Experimental Biology Meeting April, 2004).

Kim DH, Faull KF, Eckhert CD. Determination of Borate Complex with Cyclic ADP-Ribose (CADPR) by Electrospray Ionization Mass Spectrometry (ESI-MS). FASEB, 2004 (Abstract Experimental Biology Meeting April, 2004).

Concentration and Variation of Boron, Selenium and Elements Associated with Cancer Risk in Non-tumor Human Prostate Tissue

Curtis D Eckhert. Environmental Health Sciences, University of California, 650 Young Drive South, Los Angeles, California 90095-1772

Population studies of Scandinavian twins determined that 42% of the risk for prostate cancer was due to environmental exposure (Lichtenstein et al NEJM, 2000). Environmental agents that modify risk would be expected to be present at different concentrations in non-tumor than tumor tissue obtained from the same individual. Although both epidemiological and experimental studies have identified elements that modify cancer risk, few studies are available that provide elemental concentrations of human prostate tissue. The objective of the present study was to determine the concentration and variation of elements that associate with increased risk of prostate cancer (Ca and Cd), decreased risk (B, Se, Zn), and the major electrolytes of extracellular (Na) and intracellular (K) compartments. Human prostate tissue was obtained from patients (non-identifiable) following radical prostatectomy. Tissues were frozen and assigned a histological classification. Blocks of non-tumor tissue from eight different patients were analyzed by ICPMS for elemental composition. The mean concentration and standard error of mean were: ($\mu\text{mol/g} \pm \text{SEM}$) Na 60.2 ± 4.3 , K 56.6 ± 3.1 , Ca 8.8 ± 1.3 , Zn 1.5 ± 0.2 ; and ($\text{nmol/g} \pm \text{SEM}$) B 11.2 ± 2.2 , Se 2.8 ± 0.2 and Cd 0.5 ± 0.1 . This demonstrates that risk elements can be quantified in biopsy tissue for use in determining the association between local concentrations and Gleason scores of dysplasia. Funded by DOD MRMC DAMD17-03-1-0067.

Boric Acid Acts as a cADPR / RyR Antagonist During Inhibition of Human Prostate Cancer Cell Proliferation

Wade Thomas Barranco, Curtis D Eckhart, Environmental Health Sciences, University of California, 650 Young Drive South, Los Angeles, California 90095-1772

Boron reduces, in a dose dependent manner, the risk of prostate cancer (Oncology Report, In Press) and *in vivo* proliferation of human prostate cancer cells (HPCC) (EB 2003). The dominant form in serum is boric acid (BA) which can bind to NAD⁺ forming several complexes (J Mass Spec 2002). The present study used Affymetrix Human Genome U133A arrays to identify BA altered gene expression in DU-145 HPCC exposed for 24 h to 0 and 250 μ M BA (50% inhibition). Cellular calcium related genes, Calbindin-D28k and Synovial Sarcoma, X breakpoint 2 (SSX2), both decreased 2X. This led us to develop the hypothesis that BA inhibition of HPCC proliferation occurs by inhibiting intracellular Ca²⁺ mobilization. Here we report that DU145 proliferation was inhibited by BA; BAPTA (cell-permeable Ca²⁺ chelator); cADPR ryanodine receptor (RyR) antagonists: procaine, tetracaine and ruthenium red; nicotinic acid (cADPR & NAADP precursors); and diltiazem (NAADP-induced Ca²⁺ release inhibitor). These results support the hypothesis and suggest that BA binding to NAD⁺ and cADPR inhibits intracellular calcium mobilization, which in turn, inhibits cell proliferation. Funded by UC TSR&TP, DOD MRMC DAMD17-03-1-0067 and Alper Program in Environmental Genomics.

Determination of Borate Complex with Cyclic ADP-Ribose (cADPR) by Electrospray Ionization Mass Spectrometry (ESI-MS)

Danny H Kim¹, Kym F Faull², Curtis D Eckhart¹.

¹Environmental Health Sciences, University of California, 650 Young Drive South, Los Angeles, California 90095-1772, ²Pasarow Mass Spectrometry Laboratory and Psychiatry, University of California, Los Angeles, California

Electrospray ionization mass spectrometry (ESI-MS) was used to study the binding affinity of boric acid (BA) to different nucleotides (J Mass Spect 2002; EB 2002). The highest binding affinity occurred between borate and NAD⁺ with its stability dependent on nicotinamide charge and ribose phosphorylation state. The present study compared relative binding of BA to NAD⁺ and the Ca²⁺ releasing second messenger cyclic ADP-ribose (cADPR). Using flow injection analysis, samples containing 100 μ M nucleotide and 500 μ M BA were analyzed by ESI-MS in negative ion mode. Water:acetonitrile:triethylamine mixtures, 50:50:0.2 (v:v:v), at pH 10.3, were used as the solvent. The cADPR ion peak was observed at m/z 542.3. Subsequent addition of boric acid gave rise to a boric acid-cADPR complex at m/z 568.3. The binding affinity to NAD⁺ was greater than to cADPR. These results support the hypothesis that BA disrupts Ca²⁺ mobilization by binding to NAD⁺, a substrate for ADP-ribosyl cyclase, and cADPR, the major agonist of the ryanodine receptor (RyR). BA binding to these sites could be regulated by ribose dephosphorylation/phosphorylation. Funded by UC TSR&TP and US Borax